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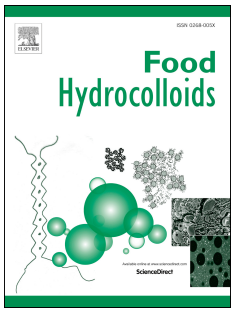

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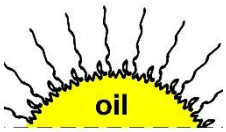
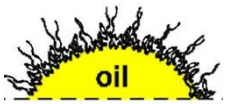
	NaCN in solution	NaCN in emulsion
Non-crosslinked		
Crosslinked		

Which casein in sodium caseinate is most resistant to *in vitro* digestion? Effect of emulsification and enzymatic structuring

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Declarations of interest: none

Abstract

We investigated the resistance of individual constituent casein epitopes (αS_1-, αS_2-, β- and κ-CN) in food-grade milk protein sodium caseinate (NaCN) to simulated human gastro-duodenal digestion. The influence of NaCN adsorption to the surface of oil-in-water emulsion droplets and the effect of crosslinking of the protein with enzyme transglutaminase (TG) on the proteolysis were studied by indirect ELISA. TG crosslinking rendered fragments of casein molecules significantly resistant to digestion. However, it depended on the type of casein and whether NaCN was presented in solution or emulsion. The crosslinking was found to considerably hinder the digestion of several amino acid regions in one of the major caseins of NaCN, β-CN. For αS_1- and αS_2-CN, only limited resistance to digestive enzymes was observed after NaCN had been crosslinked in solution but not (or to a limited extent) in emulsion. κ-CN proved to be the least resistant to the enzymatic hydrolysis regardless of the TG treatment. Our work shows for the first time how the digestibility of individual components of important food-grade protein ingredients can differ in a complex, colloidal food system. It also shows an example of how the digestibility can be modulated by chemical and physical structuring.

Keywords: Digestion; Sodium caseinate; ELISA; Emulsion; Transglutaminase; Casein

1. Introduction

Micro- and macro-structural organisations of proteins in foods are often generated by various food processing methods (e.g., emulsification, heating, gelation, enzymatic treatment, etc.). Although required to create desirable, functional structures in food, the processing can render proteins either significantly less or significantly more accessible for the digestive enzymes of the human

gastrointestinal tract and hence modify amino acid bioaccessibility during digestion (Singh & Ye, 2013; Gan, Bornhorst, Henrick, & German, 2018).

The digestion of a single protein leads to the release of hundreds of peptides in the gut lumen that can be identified by mass spectrometry (Boutrou et al., 2013) but the information is only semi-quantitative. It is therefore difficult to get a clear picture of the extent of hydrolysis of a specific protein domain (Dupont, 2017). An alternative to the mass spectrometry has been proposed based on the use of monoclonal antibodies with known specificity (Dupont, Rolet-Repecaud, & Senocq, 2003). The underlying idea is that when an antibody binds the epitope of a protein that contains a protease cleavage site, it means that the epitope has not been cleaved by the enzyme. In contrast, hydrolysis of the epitope causes a loss of interaction between the antibody and the target protein that can be easily monitored by immunoassays such as ELISA. This strategy was successfully applied to follow proteolysis events occurring during cheese ripening (Senocq, Dupont, Rolet-Repecaud, & Levieux, 2002). As a result of their loose structure, caseins (CNs) are particularly adapted to this approach as most of their epitopes are sequential, allowing the production of a wide collection of monoclonal and polyclonal antibodies targeting several epitopes of αS_1-, αS_2-, β- and κ-CN (Johansson et al., 2009).

Enzymatic crosslinking of proteins is an attractive and feasible food technology due to the specificity of enzymes and the mild reaction conditions (Buchert et al., 2010). Modification with crosslinking enzymes such as transglutaminase (TG) has been extensively used to change the functionality of proteins and thereby to improve the textural quality, stability and function of protein-based food products (Dickinson, 1997). The enzyme permanently crosslinks proteins through an acyl transfer mechanism between glutamine and lysine residues (Griffin, Casadio, & Bergamini, 2002). Monogioudi et al. (Monogioudi et al., 2011) showed that enzymatically crosslinked purified β-CN was more resistant to pepsin than a non-crosslinked protein. The crosslinking was also shown to delay the simulated human gastro-duodenal proteolysis of food-grade protein sodium caseinate (NaCN) in emulsion, which prevented the emulsion from destabilising under the gastric conditions (Macierzanka et al., 2012). Our recent *in vivo* human study (Juvonen et al., 2015) showed that even subtle structural modification of NaCN interfacial layer in emulsion by TG was able to alter the early postprandial profiles of glucose, insulin, CCK, appetite and satiety through a decreased protein digestion, without significantly affecting the gastric empting or an overall lipid digestion. Although we showed significant differences in the extent of digestion between NaCN crosslinked in emulsion and in solution (Macierzanka et al., 2012), the detailed roles of constituent casein epitopes of NaCN (i.e., αS_1-, αS_2-, β- and κ-CN) in exerting the resistance to digestion could not be evaluated. This fundamental knowledge is required for developing novel foods as the nutritional interventions aiming to modulate dietary protein bioaccessibility and amino acid bioavailability provides the best strategy for preventing diet-related health problems such as food allergies or sarcopenia.

2. Materials and methods

2.1. Materials

Food-grade sodium caseinate (NaCN; 90% protein) was obtained from DMV International (The Netherlands). Microbial transglutaminase (TG) and triglyceride oil were treated as described before (Macierzanka et al., 2012). Details have also been given in the Supplementary Material (SM; S1.1.). Eighteen monoclonal antibodies and one polyclonal antibody (SM; Table S1, Fig. S1) were taken from the INRA's collection in order to cover as much of the sequences of αS_1-, αS_2-, β- and κ-CN as possible (Johansson et al., 2009; Fig. S1). More details have been given in the SM (S1.1.).

2.2. NaCN in emulsion and solution; sample preparation and characterisation

The preparation of NaCN-stabilised emulsions and NaCN solutions, TG crosslinking, *in vitro* gastro-duodenal digestion experiments, and SDS-PAGE characterisation of the digestion samples were done as described previously (Macierzanka et al., 2012). For convenience, detailed experimental procedures have also been given in the SM.

2.3. Indirect ELISA

The indirect ELISA was performed for selected time-point samples from digestion of NaCN in order to detect protein regions (in αS_1-, αS_2-, β- and κ-CN) resistant to digestion, using the antibodies listed in Table S1. Detailed experimental procedure has been described in the SM (S1.7.)

3. Results and discussion

3.1. SDS-PAGE characterisation

We have investigated the impact of NaCN adsorption to the oil-water interface in an emulsion and its subsequent crosslinking with TG on the susceptibility of constituent casein polypeptides to simulated human gastro-duodenal proteolysis. SDS-PAGE was used initially to provide a rapid screening of the overall behaviour of NaCN during the digestion experiments carried out for the protein presented in different physical-chemical states (i.e., in solution vs. adsorbed, and non-crosslinked vs. covalently crosslinked by TG) and under different conditions (i.e., +/- vesicular PC in the gastric digestion compartment). This initial part of the study was carried out using a similar approach to the work presented previously (Macierzanka et al., 2012). Therefore, it was important to demonstrate that the SDS-PAGE characterisations of the digestion products in the present study were consistent with the results shown in that report. This offers a coherent experimental introduction to the original ELISA results reported in this paper. The SDS-PAGE results are shown in the Supplementary Material (SM; Fig. S2). Because of their consistency with the previously published work (Macierzanka et al., 2012), detailed description and discussion of the results have only been given in the SM (S2.1.).

3.2. ELISA study

An important consideration before analysing ELISA results is an effect that crosslinking might have on the binding properties of antibodies, i.e., whether the crosslinking could block antibodies even though the peptides they are specific to remain intact during the digestion. Crosslinking could theoretically affect antibody binding the target protein, causing a decrease in immunoreactivity due to steric hindrance. Nevertheless, in the present study, ELISA results were expressed as residual

immunoreactivity (RI) normalised against the immunoreactivity detected for undigested protein (native i.e. non-crosslinked, or crosslinked), thereby accounting for potential changes in antibody binding efficiency resulting from crosslinking. A loss of signal, therefore, means a hydrolysis of the epitope and not stearic hindrance.

After crosslinking NaCN with TG, significant RIs of several β-CN fragments were observed in digestion samples (Fig. 1). This suggests that the crosslinking restricted hydrolysis by digestive enzymes. The RI was significantly lower for the non-crosslinked protein. The fragment f4-28 was the only one, for which the RI of over 80% persisted until the end of the gastric phase and was still up to ca. 60% during the first 5 min of the duodenal proteolysis (Fig. 1B,D). In emulsion, approximately 70% of the adsorbed β-CN is closely associated with the oil-water interface (Mackie, Mingins, & North, 1991), with one exception being the sequence of 40–50 residues at the N-terminus. The sequence is predominantly hydrophilic and thus oriented into the aqueous phase (Dickinson, 2006). It contains four phosphoserine residues (Table S1). The electrostatic repulsion produced by this part of the protein is crucial for preventing coalescence of emulsion droplets (Caessens, Gruppen, Slangen, Visser, & Voragen, 1999). All the above suggests that the fragment f4-28 might remain exposed to the TG, not only in solution but also after the protein had been adsorbed to oil droplets in emulsion. This fragment contains one lysine (Table S1) that is the likely residue crosslinked and responsible for the high RI observed during the gastric phase of digestion (Fig. 1B,D). In the absence of crosslinking, the fragment was much more susceptible to pepsinolysis, and the RI fell to ca. 10% after 60 min of gastric digestion (Fig. 1A,C).

Another segment of β-CN, which expressed increased resistance to pepsin after crosslinking was the fragment f94-113 (Fig. 1F,H). At the end of the gastric digestion, its RI was up to ca. 40% depending on the crosslinking and digestion conditions (i.e., solution vs. emulsion, +/- PC). This short region of β-CN contains five lysine residues (Table S1) that could be crosslinked, and hence restrict access of pepsin during the digestion. However, in the absence of PC, relatively high RI (up to ca. 30% under the gastric conditions) of this fragment was also seen for the non-crosslinked protein digested in emulsion (Fig. 1G). This suggests that adsorption to the interface alone might have contributed to restricting access of pepsin. Much higher resistance to pepsinolysis (RI of ca. 95% in the absence of PC) was recorded for the adjacent fragment f133-150, regardless of the TG pre-treatment in emulsion (Fig. 1K,L), but not in solution (Fig. 1 I,J), indicating protection must have been limited to the protein segment adsorbed at the oil–water interface. Both, f133-150 and f94-113 are parts of the M_r 6 kDa peptide, which can persist during the pepsinolysis of purified β-CN in emulsion (Macierzanka et al., 2009). The f133-150 contains several aliphatic residues and a tryptophan (Table S1), which may be closely associated with the oil phase (Dickinson, Horne, Pinfield, & Leermakers, 1997). Such a close interaction of the M_r 6 kDa peptide with the oil phase was suggested to be the reason for its protection from pepsinolysis (Macierzanka et al., 2009). Here, such behaviour has been confirmed by ELISA for β-CN adsorbed to the interface in the presence of several other constituent caseins of a food-grade NaCN. In the presence of PC, the protective effect

of the interface was completely abolished for the f133-150 (Fig. 1K,L) and significantly reduced for the f94-114 (Fig. 1G), so their resistance to digestion was similar to that observed in solution (Fig. 1I,J and 1 E, respectively). Vesicular PC introduced to the gastric digestion mix is very efficient in displacing protein (including NaCN) from the oil–water interface into the surrounding aqueous phase of emulsion as the lipid is more surface active (Macierzanka et al., 2009; Macierzanka et al., 2012) After rapid desorption, the protein is then digested with the kinetics similar to those observed in solution. Here, it has been clearly seen for both f133-150 and f94-113.

The crosslinking also improved the RI of f167-178 (Fig. 1N), although to a lesser extent in emulsion (Fig. 1P). This short protein fragment contains two lysine and two glutamine residues (Table S1), which could have been crosslinked and therefore contributed to restricting the hydrolysis. Other fragments of β-CN (i.e., f33-49 and f184-202) showed very little RI (SM; Fig. S3).

We have observed a rapid degradation of αS_1-CN in non-crosslinked NaCN (Figs. 2, S4). The TG crosslinking improved resistance of two protein fragments (i.e., f56-74 and f75-92) to hydrolysis by pepsin, however the protection was predominantly observed for the protein crosslinked in solution (Fig. 2B,F) than in emulsion (Fig. 2D,H). The adsorbed αS_1-CN molecule is depicted as a tri-block polymer, with a hydrophobic region at each end and a hydrophilic central loop containing several phosphoserines (Dickinson, 2006). Thus, one can expect that in both emulsion and solution the TG should have accessed and crosslinked the central region of the protein more easily than the terminal regions. Interfacial rheology studies (Faergemand, Murray, Dickinson, & Qvist, 1999) demonstrated that the structural build-up for adsorbed αS_1-CN was slower than for either β-CN or NaCN. This was assumed to be caused by slower adsorption of αS_1-CN and/or possibly faster crosslinking of the other proteins. A significant decrease in crosslinking kinetics (calculated from the loss of monomeric caseins during the incubation with TG) upon protein adsorption to lipid droplet was found to be a general phenomenon for all constituent caseins of NaCN (Macierzanka et al., 2011). However, crosslinking of αS_1-CN was reduced much more significantly than other caseins. Hence, the limited crosslinking of adsorbed αS_1-CN might have accounted for the low RI of f56-74 and f75-92 observed here (Fig. 2D,H).

Increased RI has been recorded for another fragment of αS_1-CN, f133-151, although similar results were observed for both non-crosslinked and crosslinked samples, and only after the protein had been adsorbed at the oil–water interface (Fig. 2K,L). This segment of αS_1-CN contains 7 hydrophobic residues (i.e., Val, Ile, Met, 2× Phe, 2× Met), and was previously shown to reside very close to the interface after protein adsorption (Dickinson et al., 1997). This close interaction with the oil might have offered protection from proteolysis in a similar way as for fragments f133-150 and f94-113 of β-CN (Fig. 1G,H,K,L), although, to a more limited extent. As with the β-CN fragments, the protection was reduced when the digestion was carried out in the presence of PC (Fig. 2K,L), suggesting that also in this case PC might have displaced the protein from the oil-water interface, so the protein was digested mainly in the aqueous phase of emulsion. Other fragments of αS_1-CN (i.e., f1-19, f19-37, Nat f125-132 and f149-166) showed very little RI (SM; Fig. S4).

We have also investigated the digestibility of the two minor constituents of NaCN: αS_2-CN and κ-CN. For the digested emulsion samples, all of the αS_2-CN-specific antibodies returned very low RI, regardless of the pre-treatment with TG (data not shown). αS_2-CN is the most hydrophilic of all caseins, which is the result of three clusters of anionic groups in the amino acid sequence, composed of phosphoseryl and glutamyl residues (Farrell et al., 2004). The overall hydrophilic nature of αS_2-CN could make it more exposed to the aqueous phase of emulsion than β-CN and αS_1-CN after NaCN had been adsorbed to the oil droplets, therefore making αS_2-CN more vulnerable to the digestive enzymes. This, coupled with its lower crosslinking rate in emulsion than in solution (Macierzanka et al., 2011), would possibly explain that the limited resistance of the protein to digestion was only seen after the crosslinking in solution (Fig. S5). The most pronounced effect was observed for f96-114 (Fig. S5 F). This region of αS_2-CN contains one lysine and three glutamine residues (Table S1) that offer potential sites for TG. However, it remains unclear why the other two epitopes (f16-35 and f76-95) showed more modest resistance to digestion after the incubation with TG (Fig. S5 B,D) despite the fact that they contain 5-6 TG amino acid substrates each (Table S1).

The antibodies specific to κ-CN only showed insignificant RI of this protein in NaCN samples digested in solution or in emulsion (data not shown). The κ-CN contains lowest proportion of lysine and glutamine residues, and less phosphoserine than other caseins (Farrell et al., 2004). It also comprises a considerable amount of β-structure (Huppertz, Fox, & Kelly, 2018). Both of these factors have been used to explain much poorer crosslinking of κ-CN compared to the other caseins in NaCN (Macierzanka et al., 2011). In general, caseins in NaCN solutions exist as a dynamic system of casein monomers, complexes, and aggregates (Lucey, Srinivasan, Singh, & Munro, 2000), depending on conditions such as protein concentration, pH, ionic strength, temperature, etc. For example, at low ionic strength (3 mM) NaCN was found to be present as individual molecules (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008) but formed small aggregates (hydrodynamic radius = 11 nm) at high ionic strength (>100 mM). In dilute aqueous solutions at neutral pH, NaCN consists predominantly of protein nanoparticles (up to 20 nm) in equilibrium with free casein molecules, and some supramolecular species composed largely of κ-CN (Dickinson, 2010). Recent discussion on NaCN suspensions and casein micelles (Huppertz et al., 2017) proposed a model where NaCN particle suspension consist of assembled non-spherical primary casein particles (PCPs, which are naturally present in casein micelles). The κ-CN rich domains are likely to be located on the surface of the assembled structures. The above characteristics may reflect conditions of the NaCN solutions used in our present study. The possible easy access of digestive enzymes to κ-CN together with its poor ability to crosslinking may therefore account for the rapid hydrolysis of the protein under the *in vitro* digestion conditions.

For the αS_1-CN and the αS_2-CN, the crosslinking seemed to offer more protection to digestion after the incubation of NaCN with TG in solution than in emulsion (Figs 2 and S5). Apart from the aforementioned higher rate/degree of crosslinking of the caseins in solution (Macierzanka et al., 2011), the reason might also lie in the type of the crosslinking observed in the two systems. In the same studies, it was shown that incubation of NaCN with TG in solution might have led to some

intramolecular crosslinking as the oligomers formed were found to be more mobile on SDS-PAGE than their counterparts formed from NaCN crosslinked at the oil−water interface, indicating that intermolecular crosslinking might have prevailed at the interface. Therefore, the higher extent of crosslinking and more compact structuring of the proteins offered by the intramolecular crosslinks might account for some fragments of αS_1-CN and αS_2-CN incubated with TG in solution being more resistant to digestion than those crosslinked in emulsion. This leads to the conclusion that the group of oligomers of M_r ca. 50-100 kDa formed during the gastric digestion of crosslinked NaCN in emulsion (Fig. S2 F), might have been mainly composed of the β-CN fragments that showed significant resistance to pepsinolysis (Fig. 1).

Our results suggest that the TG crosslinking can improve resistance of casein molecules to gastrointestinal digestion, if, for example, this is required for modulating phase behaviour of protein-stabilised emulsions in the stomach and the rate of nutrients release (van Aken et al., 2011). The findings might then be useful for optimising protein structuring in personalised nutrition in order to modulate specific physiological responses to food, such as the ileal brake, which could in turn determine satiety and calorie intake.

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Supplementary Material

Detailed description of the materials and methods used as well as additional data and discussion of the results obtained.

References

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Benamouzig, R., Tome, D., & Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *American Journal of Clinical Nutrition, 97,* 1314-1323.

Buchert, J., Ercili Cura, D., Ma. H., Gasparetti, C., Monogioudi, E., Faccio, G., Mattinen, M., Boer, H., Partanen, R., Selinheimo, E., Lantto, R., & Kruus, K. (2010) Crosslinking food proteins for improved functionality. *Annual Review of Food Science and Technology, 1,* 113–138.

Caessens, P. W. J. R., Gruppen, H., Slangen, C. J., Visser, S., & Voragen, A. G. J. (1999). Functionality of β-casein peptides: Importance of amphipathicity for emulsion-stabilizing properties. *Journal of Agricultural and Food Chemistry, 47,* 1856–1862.

Dickinson, E. (1997). Enzymatic crosslinking as a tool for food colloid rheology control and interfacial stabilization. *Trends in Food Science and Technology, 8,* 334-339.

Dickinson, E. (2006). Colloid science of mixed ingredients. *Soft Matter, 2,* 642–652.

Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces,* 81, 130–140.

Dickinson, E., Horne, D. S., Pinfield, V. J., & Leermakers, F. A. M. (1997). Self-consistent-field modelling of casein adsorption. Comparison of results for α_{s1}-casein and β-casein. *Journal of the Chemical Society, Faraday Transactions, 93,* 425-432.

Dupont, D. (2017). Peptidomic as a tool for assessing protein digestion. *Current Opinion in Food Science, 16,* 53-58.

Dupont, D., Rolet-Repecaud, O., & Senocq, D. (2003). A new approach to monitoring proteolysis phenomena using antibodies specifically directed against the enzyme cleavage site on its substrate. *Analytical Biochemistry, 317,* 240-246.

Faergemand, M., Murray, B. S., Dickinson, E., & Qvist, K. B. (1999). Cross-linking of adsorbed casein films with transglutaminase. *International Dairy Journal, 9,* 343-346.

Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C. L., Hollar, C. M., Ng-Kwai-Hang, K. F., & Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk - Sixth revision. *Journal of Dairy Science, 87,* 1641-1674.

Gan, J. A., Bornhorst, G. M., Henrick, B.M., & German, J.B. (2018) Protein digestion of baby foods: Study approaches and implications for infant health. *Molecular Nutrition & Food Research, 62,* Article no: 1700231

Griffin, M. A., Casadio, R., & Bergamini, C. M. (2002). Transglutaminases: nature's biological glues. *Biochemical Journal, 368,* 377-396.

HadjSadok, A., Pitkowski, A., Nicolai, T., Benyahia, L., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids, 2*2, 1460-1466

Huppertz, T., Gazi, I., Luyten, H., Nieuwenhuijse, H., Alting, A., & Schokker, E. (2017). Hydration of casein micelles and caseinates: Implications for casein micelle structure. *International Dairy Journal, 74,* 1-11.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2018). The caseins: Structure, stability, and functionality. In R. Y. Yada (Ed.), *Proteins in Food Processing (Second Edition)* (pp. 49-92). Duxford: Woodhead Publishing.

Johansson, A., Lugand, D., Rolet-Repecaud, O., Molle, D., Delage, M. M., Peltre, G., Marchesseau, S., Leonil, J., & Dupont, D. (2009). Epitope characterization of a supramolecular protein assembly with a collection of monoclonal antibodies: The case of casein micelle. *Molecular Immunology, 46,* 1058-1066.

Juvonen, K. R., Macierzanka, A., Lille, M. E., Laaksonen, D. E., Mykkänen, H. M., Niskanen, L. K., Pihlajamäki, J., Mäkelä, K., Mills, C. E. N., Mackie, A. R., Malcolm, P., Herzig, K.-H., Poutanen, K. S., & Karhunen, L. J. (2015). Cross-linking of sodium caseinate structured emulsion with transglutaminase alters the postprandial metabolism and appetite responses in healthy young individuals. *British Journal of Nutrition, 114,* 418–429.

Lucey, J. A., Srinivasan, M., Singh, H., & Munro, P. A. (2000). Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *Journal of Agricultural and Food Chemistry, 48,* 1610-1616.

Macierzanka, A., Sancho, A. I., Mills, E. N. C., Rigby, N. M., & Mackie, A. R. (2009). Emulsification alters simulated gastrointestinal proteolysis of β-casein and β-lactoglobulin. *Soft Matter, 5,* 538–550.

Macierzanka A., Bordron F., Rigby N. M., Mills E. N. C., Lille M., Poutanen K., & Mackie A. R. (2011). Transglutaminase cross-linking kinetics of sodium caseinate is changed after emulsification. *Food Hydrocolloids, 25,* 843-850.

Macierzanka, A., Böttger, F., Rigby, N. M., Lille, M., Poutanen, K., Mills, E. N. C., & Mackie A. R. (2012). Enzymatically structured emulsions in simulated gastrointestinal environment: Impact on interfacial proteolysis and diffusion in intestinal mucus. *Langmuir, 28,* 17349–17362.

Mackie, A. R., Mingins J., & North, A. N. (1991). Characterisation of adsorbed layers of a disordered coil protein on polystyrene latex. *Journal of the Chemical Society, Faraday Transactions, 87,* 3043–3049.

Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J., & Mattinen, M.-L. (2011). Effect of enzymatic cross-linking of β-casein on proteolysis by pepsin. *Food Hydrocolloids, 25,* 71-81.

Senocq, D., Dupont, D., Rolet-Repecaud, O., & Levieux, D. (2002). ELISA for monitoring the cleavage of beta-casein at site Lys(28)-Lys(29) by plasmin during Comte cheese ripening. *Journal of Dairy Research, 69,* 491-500.

Singh, H., & Ye, A. Q. (2013). Structural and biochemical factors affecting the digestion of protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 18,* 360-370.

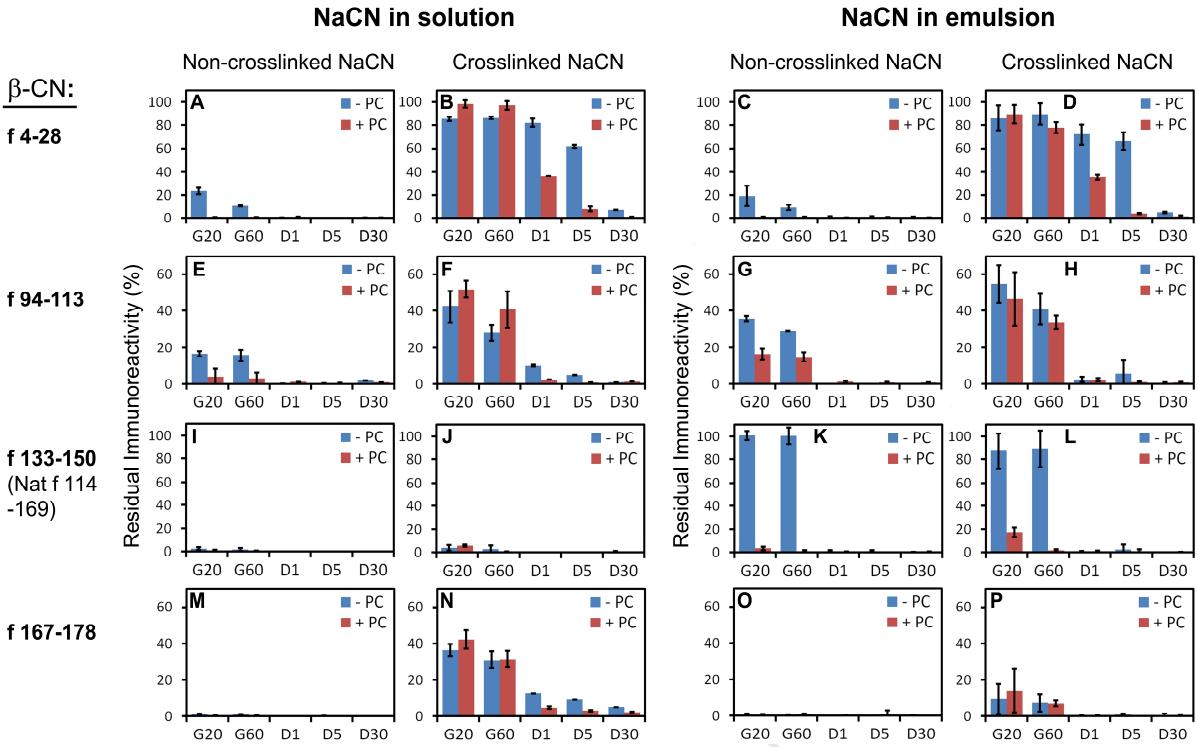
van Aken, G. A., Bomhof, E., Zoet, F. D., Verbeek, M., Oosterveld, A. (2011). Differences in in vitro gastric behaviour between homogenized milk and emulsions stabilised by Tween 80, whey protein, or whey protein and caseinate. *Food Hydrocolloids, 25,* 781−788.

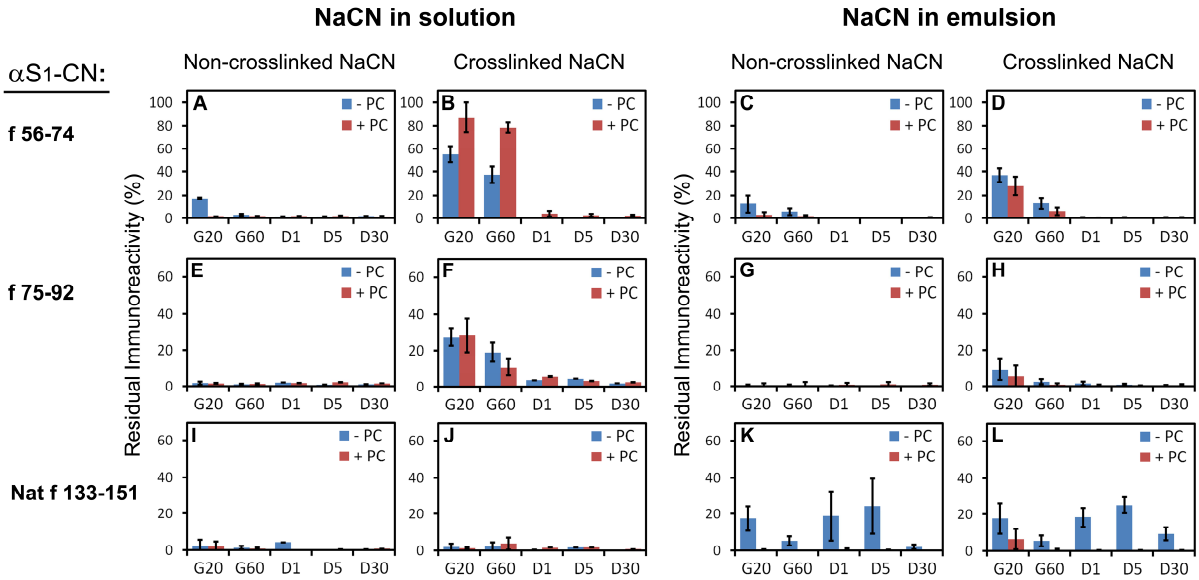
Figure captions

Fig. 1. Residual immunoreactivity (RI) of β-CN fragments (f) determined in time-point samples collected during the *in vitro* digestion of NaCN (results were normalised against the immunoreactivity detected for undigested protein sample; native i.e. non-crosslinked, or crosslinked). Effect of (i) presenting NaCN in aqueous solution (1 mg/mL) or emulsion (1 mg/mL), (ii) crosslinking of the protein with TG before digestion, and (iii) carrying out the digestion experiments in the presence or absence of vesicular phosphatidylcholine (PC) in the gastric phase of digestion. Gastric samples have been marked with G and duodenal with D, followed by a number corresponding to the digestion

time (min) after which the samples were taken. Extended version of Fig. 1 has been shown in the Supplementary Material (Fig. S3).

Fig. 2. Residual immunoreactivity (RI) of αS_1-CN fragments (f) determined in time-point samples collected during the *in vitro* digestion of NaCN. For more details see caption of Fig. 1. Extended version of Fig. 2 has been shown in the Supplementary Material (Fig. S4).





Highlights:

- Transglutaminase crosslinking can impact on gastrointestinal proteolysis
- The crosslinking improves resistance to digestion of caseins in sodium caseinate
- The resistance strongly depends on the type of constituent casein (αS_1, αS_2, β, κ)
- The resistance depends on presenting protein in either solution or emulsion